Exhibit 69

Gynecologic Oncology 135 (2014) 297–304

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Pre-diagnostic serum levels of inflammation markers and risk of ovarian cancer in the Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial☆

Britton Trabert [a,*], Ligia Pinto [a,b], Patricia Hartge [a], Troy Kemp [b], Amanda Black [a], Mark E. Sherman [c], Louise A. Brinton [a], Ruth M. Pfeiffer [a], Meredith S. Shiels [a], Anil K. Chaturvedi [a], Allan Hildesheim [a], Nicolas Wentzensen [a]

[a] Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA
[b] HPV Immunology Laboratory, Frederick National Laboratory for Cancer Research, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Frederick, MD, USA
[c] Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

HIGHLIGHTS

- We evaluated 46 pre diagnostic inflammation related biomarkers and ovarian cancer.
- CRP, TNF α, and IL 8 are associated with increased risk of subsequently developing ovarian cancer.
- Increased risks with CRP and TNF α are apparent 5 or more years prior to diagnosis.
- Our study provides additional evidence that inflammation plays an important role in ovarian carcinogenesis.

ARTICLE INFO

Article history:
Received 21 June 2014
Accepted 17 August 2014
Available online 23 August 2014

Keywords:
Ovarian cancer
Nested case–control study
Pre-diagnostic
Inflammation
Circulating inflammation markers
Cytokines

ABSTRACT

Objective. Pro inflammatory mechanisms may explain the increased ovarian cancer risk linked to more life time ovulations, endometriosis, and exposure to talc and asbestos, as well as decreased risk with non steroidal anti inflammatory drugs. Limited data are available to estimate ovarian cancer risk associated with levels of circulating inflammatory markers.

Methods. We conducted a nested case control study within the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. Pre diagnostic serum levels of 46 inflammation related biomarkers (11 with a priori hypotheses; 35 agnostic) were measured in 149 incident ovarian cancer cases and 149 matched controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using conditional logistic regression and adjusted for identi fied covariates.

Results. Increased ovarian cancer risk was associated with elevated levels of C reactive protein (CRP) [tertile (T)3 vs. T1: OR (95% CI) 2.04 (1.06 3.93), p trend = 0.03], interleukin (IL) 1α [detectable vs. undetectable: 2.23 (1.14 4.34)] and tumor necrosis factor alpha (TNF α) [T3 vs. T1: 2.21 (1.06 4.63), p trend = 0.04]. Elevated IL 8 was non significantly associated with risk [T3 vs. T1: 1.86 (0.96 3.61), p trend = 0.05]. In analyses restricted to serous ovarian cancer (n = 83), the associations with CRP and IL 8 remained or strengthened [CRP T3 vs. T1: 3.96 (1.14 11.14), p trend = 0.008; IL 8 T3 vs. T1: 3.05 (1.09 8.51), p trend = 0.03]. Elevated levels of CRP and TNF α remained positively associated with ovarian cancer risk in analysis restricted to specimens collected at least 5 years before diagnosis (n = 56).

Conclusion. These results suggest that CRP, IL 1α, IL 8, and TNF α are associated with increased risk of subse quently developing ovarian cancer.

Published by Elsevier Inc.

Introduction

Epidemiologic evidence implicates chronic inflammation as a central mechanism in the pathogenesis of ovarian cancer, the most lethal gynecologic cancer among women in the United States [1]. Chronic

☆ Financial support: This research was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics and by contracts from the Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, U.S. Department of Health and Human Services.

* Corresponding author at: 9609 Medical Center Drive, Room 7E-228, Bethesda, MD 20892-9774, USA. Fax: +1 240 276 7838.
E-mail address: britton.trabert@nih.gov (B. Trabert).

http://dx.doi.org/10.1016/j.ygyno.2014.08.025
0090-8258/Published by Elsevier Inc.

inflammation can induce rapid cell division, increasing the possibility for replication error, ineffective DNA repair and subsequent mutation. Ovarian cancer has been linked to several events and conditions which are related to inflammation and repair, including incessant ovulation, endometriosis, exposure to talc and asbestos, and in some studies pelvic inflammatory disease [reviewed in [2]]. In addition, reduced risks found for aspirin use [3] could be related to direct anti inflammatory actions, while reduced risks related to tubal ligation and hysterectomy could reflect limited exposure to environmental causes of inflammation [2]. Understanding the role of inflammation in ovarian cancer etiology is complicated by growing recognition that there are least two main types of these tumors, which differ clinically and biologically [4]. Increasing evidence suggests that some high grade serous carcinomas, the most common and lethal subtype, arise from the fimbria of the fallopian tube rather than the ovarian surface epithelium [4].

Recent clinical and prospective data suggest that C reactive protein (CRP), a marker of global inflammation, is associated with increased ovarian cancer risk [5 8]. Pre diagnostic CRP levels have been associated with ovarian cancer risk in all four studies [5 8] evaluating the association; with one study showing an association only among women with "clinically high" CRP levels (>10 mg/L vs. <1 mg/L) [6].

Other inflammatory markers may be important in ovarian carcinogenesis. In premenopausal women ovarian epithelial cells secrete cytokines as part of ovarian function and some of these cytokines are also produced by ovarian cancer cells [9 11]. Follicle rupture during ovulation involves tissue remodeling with high cell turnover that is characteristic of inflammatory reactions. Many inflammatory mediators, including prostaglandins, leukotrienes, and cytokines, are locally elevated during ovulation [12]. Epithelial cells in proximity to ovulating follicles are likely exposed to these inflammatory mediators that may signal oxidative stress, and enhance the risk of mutagenesis. In addition, data from animal and limited human studies supports the hypothesis that ovulation may trigger cellular events that result in carcinogenesis [13,14]. Importantly, cytokines involved in ovarian function, follicle rupture, and repair (physiologic processes before menopause) are suggested to remain activated in postmenopausal women and may play an etiologic role in ovarian carcinogenesis; these cytokines include: interleukins (IL) 1α, IL 1β, IL 2, IL 6, IL 8, IL 10, tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), granulocyte colony stimulating factor (G CSF), and granulocyte macrophage colony stimulating factor (GM CSF) [11].

To gain a better understanding of the etiologic role of inflammation markers in ovarian cancer development, we conducted a nested case control study within the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. We used multiplexed inflammatory maker panels to measure 46 inflammation related markers, including several inflammation markers with existing evidence of associations with ovarian function or ovarian cancer risk.

Materials and methods

Study design

We conducted a nested case control study within the screening arm of the PLCO Cancer Screening Trial. Details of the screening trial have been reported previously [15]. Briefly, between 1993 and 2001, approximately 155,000 subjects (78,216 women) 55 74 years of age were recruited from ten cities from the general population and randomized to the screening or non screening arm of the study. Screening arm subjects provided blood samples at baseline and five subsequent annual medical examinations. Samples were processed and frozen within 2 h of collection, and stored at −70 °C [16]. In addition to trial cancer outcomes (prostate, lung, colorectal and ovarian cancers) detected by annual screening examinations during the first six years of follow up, individuals were followed for all cancer diagnoses by annual mailed questionnaires. All cancer diagnoses were pathologically confirmed through medical record abstraction. Institutional review boards of the U.S. National Cancer Institute and the ten study centers approved the trial, and all participants provided written informed consent. The nested case control study was also approved by the institutional review board of the National Cancer Institute.

We identified 150 first primary ovarian cancer cases diagnosed between two and fourteen years after blood collection from among the eligible screening arm participants followed through December 31, 2008. Eligibility criteria included the availability of an unthawed serum sample, consent to biochemical studies, completion of the baseline questionnaire, and no history of cancer (other than non melanoma skin cancer) prior to ovarian cancer diagnosis. Serum specimens from a single visit were measured for each study subject. To ensure a relatively equal distribution of specimens between 2 and 14 years prior to diagnosis, 11.4% of samples selected were measured at baseline and the remaining at follow up visits (18.1% year 1, 26.2% year 2, 12.8% year 4, and 31.5% year 5). Controls were individually matched to cases on the basis of age at blood collection (55 59, 60 64, 65 69, 70+ years), race (white, black, other), study center, and time (a.m., p.m.) and date (three month categories) of blood collection. Controls were restricted to women with no history of oophorectomy at the time of diagnosis of their matched case. We were unable to identify a suitable matched control for one case, therefore our final analytic sample consisted of 149 cases and 149 matched controls.

Laboratory methods

We measured circulating levels of 60 immune and inflammation markers, including cytokines, chemokines, growth factors, and soluble products of immune activation (Supplemental Table 1). Assays for these markers have demonstrated satisfactory performance and reproducibility [17] and include assessment of 11 markers linked with either ovulation or ovarian cancer risk. Fifty nine of the 60 markers were measured on four Luminex bead based commercial assay panels (Millipore Inc., Billerica, MA). The remaining marker, CRP, was measured with a Luminex bead based assay from Millipore (Billerica, MA) and tested according to the manufacturer's protocol. Batched assays were performed in a single laboratory (LP). Concentrations of the 60 multiplexed markers were calculated using a four or five parameter logistic curve using Bioplex Manager 6.1 software (BioRad, Hercules, CA). Cases and matched controls were included in the same analytic batch. Samples were assayed in duplicate and averaged to calculate concentrations. To evaluate assay performance we included a replicate sample from a quality control (QC) pool in each batch. Percent detected above the lower limit of detection (LLOD), coefficients of variation (CVs), and intraclass correlation coefficients (ICCs) for the QC samples of all measured inflammation markers are summarized in Supplemental Table 1. We excluded from further study 14 markers with <20% of values above the LLOD. Although IL 1α had only 18.4% of values above the LLOD we included this marker in analyses because it was one of eleven markers with a priori hypothesis regarding a potential ovarian cancer association and it was close to the 20% threshold. After these exclusions, 46 markers were included in the statistical analysis.

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between the serum inflammation markers and ovarian cancer risk were calculated using conditional logistic regression models. All models were adjusted for a priori potential confounding factors: parity (nulliparous, parous), duration of oral contraceptive use (never, 1 5 years, 6+ years), duration of menopausal hormone therapy use (never, 1 5 years, 6+ years), cigarette smoking status (never, former, current) and body mass index (BMI; <25, 25 29.9, 30+ kg/m^2). Further adjustment by aspirin or ibuprofen use, or family history of breast or ovarian cancer, did not substantially change the observed effect estimates, therefore we did not include these covariates in the model.

Marker levels were categorized into groups based on the proportion of individuals with measurements above the LLOD as follows: markers with 66% of individuals with measurements above LLOD or greater (n = 26) were categorized into tertiles based on the distribution among controls, individuals with values at or below LLOD were included in the lowest tertile; and markers with fewer than 66% of individuals with measurements above LLOD were categorized into two groups (detectable vs. non detectable (≤LLOD)). To compute tests for trend across tertile categories, intra category medians were modeled as a continuous parameter. Q values which reflect the false discovery rate (FDR) were calculated to account for multiple comparisons.

In secondary analyses, we evaluated associations stratified by serous/non serous histologic subtype as well as time between blood collection and diagnosis (2 <5 years and 5 14 years). Given the modest correlation between the markers, we further evaluated those markers that were associated with ovarian cancer risk in a mutually adjusted model. For the analysis of CRP, we conducted a sensitivity analysis excluding individuals who reported current use of menopausal hormone therapy at blood draw, as a high CRP level in women taking hormone therapy may be due to a first pass effect [18]. We also conduct ed a sensitivity analysis excluding individuals with known inflammato ry conditions: cardiovascular disease, rheumatoid arthritis, and diabetes (n = 146). We further examined associations modeling the cross classification of the inflammatory marker and its modulator (e.g. TNF α and its receptor TNF α R1). Finally, given that cancer antigen (CA) 125 is currently the best predictor of ovarian cancer we evaluated the correlation between CA 125 and the inflammatory marker level from the same study year. Correlation coefficients for the markers evaluated were non significant and less than 0.15 (results not shown). Further, only 5 subjects were classified as CA 125 positive at the corresponding study year of blood draw, therefore further model adjustment for CA 125 was uninformative.

Table 1
Demographic and health characteristics of cases and controls, nested case–control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	Cases (n = 149)		Controls (n = 149)	
	Mean	SD	Mean	SD
Age at baseline	63.2	5.5	63.0	5.3
	n[a]	%	n[a]	%
Race				
Non-Hispanic White	138	92.6	138	92.6
Non-Hispanic Black	5	3.4	5	3.4
Hispanic	3	2.0	3	2.0
Asian	3	2.0	3	2.0
Highest education level attained				
High school or less	39	26.2	47	31.5
Some post-high school training	54	36.2	49	32.9
College graduate	56	37.6	53	35.6
Body mass index (kg/m2)				
<25	65	43.6	64	43.0
25–29.9	54	36.2	53	35.6
≥30	28	18.8	32	21.5
Cigarette smoking status				
Never	80	53.7	95	63.8
Current	12	8.1	15	10.1
Former	57	38.3	39	26.2
Parity				
Nulliparous	10	6.7	5	3.4
Parous	139	93.3	144	96.6
Duration of oral contraceptive use				
Never	80	53.7	73	49.0
1–5 years	46	30.9	48	32.2
6+ years	23	15.4	28	18.8
Duration of menopausal hormone therapy use[b]				
Never	39	26.2	59	39.6
1–5 years	48	32.2	47	31.5
6+ years	62	41.6	43	28.9

[a] Values may not sum to total because of missing data.
[b] Frequency of duration of menopausal hormone therapy use was differed between cases and controls, p-value < 0.05.

Results

The distribution of selected demographic and health characteristics of the cases and controls is summarized in Table 1. Participants were on average 63 years old at enrollment and were predominately white (92.6%). The median length of follow up from blood collection to case diagnosis was 4.2 years (interquartile range (IQR): 2.8 6.7 years). The median length of follow up from blood collection until the end of follow up for controls was 9.9 years (IQR: 8.0 12.9).

Of the eleven markers with an a priori hypothesis regarding a poten tial ovarian cancer association (CRP, IL 1α, IL 1β, IL 2, IL 6, IL 8, IL 10, TNF α, IFN γ, G CSF, and GM CSF), four were positively associated with ovarian cancer risk in the current study (Table 2): CRP [tertile (T) 3 vs. T1: OR (95% CI) 2.04 (1.06 3.93), p trend = 0.03], IL 1α [detectable vs. undetectable: 2.23 (1.14 4.34)], TNF α [T2 vs. T1: 1.89 (1.01 3.53), T3 vs. T1: 2.21 (1.06 4.63), p trend = 0.04] and IL 8 [T3 vs. T1: OR 95% CI 1.86 (0.96 3.61), p trend = 0.05]. The association with IL 1α is based on 34 exposed cases only and should be interpreted with caution. In analyses restricted to serous ovarian tumors (n = 83), the associations with CRP, IL 1α, and IL 8 remained [CRP T3 vs. T1: OR (95% CI) 3.96 (1.14 11.14), p trend = 0.008; IL 1α detectable vs. non detectable: OR (95% CI) 2.70 (1.10 6.36); IL 8 T3 vs. T1: OR (95% CI) 3.05 (1.09 8.51), p trend = 0.03] (Table 3). The association for serous tumors with TNF α was no longer statistically significant [T3 vs. T1: OR 95% CI 2.06 (0.71 6.00), p trend = 0.19]; however TNF α was associated with an increased risk in analyses restricted to non serous ovarian tumors (n = 76) [T2 vs. T1: 4.92 (1.52 15.90), T3 vs. T1: 4.36 (1.11 17.05), p trend = 0.05]. After correction for multiple comparisons, CRP was significantly associated with serous ovarian can cer at FDR less than 0.10. The q values for the associations between CRP, IL 1α, TNF α, IL 8 and ovarian cancer risk were 0.13. The q values for the remaining associations in Tables 2 and 3 were all greater than 0.13.

Of the remaining 35 markers with weak or no prior evidence of an association (Supplemental Tables 2 and 3), three were positively associ ated with ovarian cancer risk. Among the markers with 66% of individ uals with measurements above the LLOD (Supplemental Table 2), interferon gamma induced protein 10 (IP 10) and macrophage inflam matory protein 1beta (MIP 1β) were associated with increased ovarian cancer risk comparing the second tertile to the first tertile; however, the trend across tertiles and the association comparing the third tertile to the first tertile were not statistically significant. Among markers with fewer than 66% of individuals with measurements above the LLOD (Supplemental Table 3), fibroblast growth factor 2 (FGF 2) was associ ated with increased risk [detectable vs. ≤LLOD FGF 2: OR (95% CI) 2.21 (1.15 4.25)]; however, this result should be interpreted with cau tion, given that it is based on 28 exposed cases. The remaining markers evaluated were not associated with increased or decreased ovarian cancer risk (Supplemental Tables 2 and 3, and Fig. 1). The q values for all markers evaluated in Supplemental Tables 2 and 3 were all >0.10.

In analyses restricted to cases with specimens collected at least five years prior to diagnosis (n = 56), CRP and TNF α levels remained pos itively associated with ovarian cancer risk [CRP T3 vs. T1: OR (95% CI) 4.51 (1.08 18.82), p trend = 0.03; TNF α T3 vs. T1: OR (95% CI) 5.55 (1.19 25.83), p trend = 0.04] (results not tabled), while the trend across tertiles for IL 8 was no longer statistically significant [T3 vs. T1: OR (95% CI) 1.70 (0.55 5.27), p trend = 0.34] (results not tabled). Increased risk of ovarian cancer with IP 10 and FGF 2 remained in analyses restricted to specimens collected at least five years prior to diagnosis (results not shown).

In mutually adjusted models there was an independent association between CRP and ovarian cancer risk in the analysis of all cases

Table 2
Associations of a priori selected pre-diagnostic circulating inflammation markers and ovarian cancer risk, nested case–control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	Cases (n = 149)		Controls (n = 149)			
	n	%	n	%	OR[a]	95% CI
C-reactive protein (CRP) (mg/L)						
<3.23	39	26.2	49	32.9	1.00	Ref
3.23–9.76	47	31.5	50	33.6	1.29	0.68–2.41
>9.76	63	42.3	50	33.6	2.04	1.06–3.93
p-trend					0.03	
Interleukin (IL)-1α (ng/L)[b]						
≤LLOD (3.2)	115	77.2	128	85.9	1.00	Ref
Detectable	34	22.8	21	14.1	2.23	1.14–4.34
IL-1β (ng/L)						
≤LLOD (0.64)	110	73.8	111	74.5	1.00	Ref
Detectable	39	26.2	38	25.5	1.06	0.58–1.94
IL-2 (ng/L)						
≤LLOD (0.64)	112	75.2	119	79.9	1.00	Ref
Detectable	37	24.8	30	20.1	1.50	0.79–2.84
IL-6 (ng/L)						
≤LLOD (0.64)	100	67.1	108	72.5	1.00	Ref
Detectable	49	32.9	41	27.5	1.41	0.81–2.46
IL-8 (ng/L)						
<1.87	41	27.5	49	32.9	1.00	Ref
1.87–3.79	43	28.9	50	33.6	1.17	0.60–2.27
>3.79	65	43.6	50	33.6	1.86	0.96–3.61
p-trend					0.05	
IL-10 (ng/L)						
≤LLOD (0.64)	104	69.8	111	74.5	1.00	Ref
Detectable	45	30.2	38	25.5	1.39	0.77–2.50
Interferon gamma (IFN-γ) (ng/L)						
≤LLOD (3.2)	116	77.9	119	79.9	1.00	Ref
Detectable	33	22.2	30	20.1	1.68	0.87–3.27
Granulocyte colony-stimulating factor (G-CSF) (ng/L)						
≤LLOD (16.0)	82	55.0	86	57.7	1.00	Ref
Detectable	67	45.0	63	42.3	1.20	0.72–2.01
Granulocyte colony-stimulating factor (GM-CSF) (ng/L)						
≤LLOD (13.2)	86	57.7	91	61.1	1.00	Ref
Detectable	63	42.3	58	38.9	1.25	0.74–2.11
Tumor necrosis factor alpha (TNF-α) (ng/L)						
<4.05	36	24.2	49	32.9	1.00	Ref
4.05–5.48	59	39.6	50	33.6	1.89	1.01–3.53
>5.48	54	36.2	50	33.6	2.21	1.06–4.63
p-trend					0.04	

[a] Conditional logistic regression models adjusted for body mass index, cigarette smoking status, parity, duration of oral contraceptive use, and duration of menopausal hormone therapy use.
[b] Less than 20% of marker values were above lower limit of detection, results should be interpreted with caution.

(Table 4). In analyses restricted to serous tumors the increased risk with elevated serum levels of IL 8 and CRP remained in the mutually adjust ed model, whereas in the analysis of specimens collected at least five years prior to cancer diagnosis both CRP and TNF α were independently associated with increased risk. In contrast, in analyses restricted to specimens collected less than 5 years prior to diagnosis, ORs from the mutually adjusted model were not significantly elevated for CRP, IL 8 or TNF α. Further, the increased risk of ovarian cancer with elevated CRP was not attenuated in an analysis restricted to women who did not report menopausal hormone use at the time of blood draw [OR T3 vs. T1 = 2.21] (results not tabled). Results were not substantially atten uated after excluding cases and controls with cardiovascular disease, rheumatoid arthritis, and diabetes (results not shown). Finally, there were no statistically significant associations based on analyses modeling the cross classification of the inflammatory marker and its modulator (results not shown).

Discussion

We identified several circulating inflammation markers that were associated with risk of developing ovarian cancer between 2 and 14 years later. Specifically we observed associations between elevated CRP, IL 1α, IL 8, and TNF α and risk of epithelial ovarian cancer in a nested case control study in the PLCO Cancer Screening Trial. For CRP and TNF α, we found the same effects for serum samples collected 5 or more years prior to diagnosis, supporting that reverse causation does not explain the effect.

Data from animal and limited human studies support the hypothesis that ovulation may trigger cellular events that result in carcinogenesis. Hyperovulatory hens have markedly increased likelihood of developing ovarian adenocarcinomas, as do rats with hyper proliferating ovarian epithelial cells [13,14]. It is plausible that cytokines play a role in the development of pre neoplastic cells in the epithelium that, under con tinuous cytokine stimulation, progress to cancer cells, suggesting that elevated levels of these cytokines may confer increased ovarian cancer risk [9 11]. Further, it has been shown that ovarian epithelial cells se crete cytokines and that these same factors are also produced by ovarian cancer cells further supporting that the recruitment of normally secret ed cytokines into dysregulated autocrine loops may be important in neoplastic progression [9,10].

Our results further support the association between pre diagnostic CRP levels and ovarian cancer observed in four previous studies [5 8]. CRP is a marker of global inflammation that has been associated with other cancers. It is not clear whether CRP directly influences ovarian car cinogenesis or is an indirect marker of inflammatory exposures to the ovary. One study suggested that high levels of CRP in ovarian cancer

Table 3
Associations of a priori selected pre-diagnostic circulating inflammation markers and ovarian cancer risk by serous and non-serous histology, nested case–control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	Serous cases		Controls				Non-serous cases		Controls			
	N = 83		N = 88		OR[a]	95% CI	N = 66		N = 76		OR[b]	95% CI
CRP (mg/L)												
<3.23	20	24.1	31	35.2	1.00	Ref	19	28.8	26	34.2	1.00	Ref
3.23–9.76	26	31.3	31	35.2	1.68	0.60–4.74	21	31.8	23	30.3	1.76	0.67–4.60
>9.76	37	44.6	26	29.6	3.96	1.41–11.14	26	39.4	27	35.5	2.13	0.75–6.05
p-trend					0.008						0.22	
IL-1α (ng/L)[c]												
≤LLOD (3.2)	61	73.5	75	85.2	1.00	Ref	54	81.8	65	85.5	1.00	Ref
Detectable	22	26.5	13	14.8	2.70	1.10–6.63	12	18.2	11	14.5	2.12	0.66–6.88
IL-1β (ng/L)												
≤LLOD (0.64)	58	69.9	64	72.7	1.00	Ref	52	78.8	60	79.0	1.00	Ref
Detectable	25	30.1	24	27.3	1.28	0.52–3.18	14	21.2	16	21.1	1.34	0.54–3.36
IL-2 (ng/L)												
≤LLOD (0.64)	59	71.1	68	77.3	1.00	Ref	53	80.3	64	84.2	1.00	Ref
Detectable	24	28.9	20	22.7	1.99	0.80–4.99	13	19.7	12	15.8	1.73	0.62–4.76
IL-6 (ng/L)												
≤LLOD (0.64)	54	65.1	64	72.7	1.00	Ref	46	69.7	57	75.0	1.00	Ref
Detectable	29	34.9	24	27.3	1.72	0.76–3.91	20	30.3	19	25.0	1.67	0.68–4.07
IL-8 (ng/L)												
<1.87	22	26.5	33	37.5	1.00	Ref	19	28.8	23	30.3	1.00	Ref
1.87–3.79	23	27.7	26	29.6	1.61	0.57–4.52	20	30.3	30	39.5	0.68	0.26–1.78
>3.79	38	45.8	29	33.0	3.05	1.09–8.51	27	40.9	23	30.3	1.45	0.47–4.54
p-trend					0.03						0.33	
IL-10 (ng/L)												
≤LLOD (0.64)	56	67.5	65	73.9	1.00	Ref	48	72.7	58	76.3	1.00	Ref
Detectable	27	32.5	23	26.1	2.12	0.84–5.36	18	27.3	18	23.7	1.57	0.64–3.87
IFN-γ (ng/L)												
≤LLOD (3.2)	67	80.7	69	78.4	1.00	Ref	49	74.2	64	84.2	1.00	Ref
Detectable	16	19.3	19	21.6	1.07	0.46–2.48	17	25.8	12	15.8	4.42	1.21–16.11
G-CSF (ng/L)												
≤LLOD (16.0)	50	60.2	53	60.2	1.00	Ref	32	48.5	44	57.9	1.00	Ref
Detectable	33	39.8	35	39.8	1.44	0.67–3.10	34	51.5	32	42.1	1.23	0.57–2.63
GM-CSF (ng/L)												
≤LLOD (13.2)	52	62.7	54	61.4	1.00	Ref	34	51.5	45	59.2	1.00	Ref
Detectable	31	37.4	34	38.6	1.33	0.62–2.82	32	48.5	31	40.8	1.44	0.63–3.26
TNF-α (ng/L)												
<4.05	24	28.9	28	31.8	1.00	Ref	12	18.2	26	34.2	1.00	Ref
4.05–5.48	26	31.3	30	34.1	1.13	0.49–2.59	33	50.0	25	32.9	4.92	1.52–15.90
>5.48	33	39.8	30	34.1	2.06	0.71–6.00	21	31.8	25	32.9	4.36	1.11–17.05
p-trend					0.19						0.047	

[a] Conditional logistic regression models adjusted for body mass index, cigarette smoking status, parity, duration of oral contraceptive use, and duration of menopausal hormone therapy use.
[b] Some matched sets include more than 1 case–control pair; therefore, in conditional logistic regression analyses restricted to specific tumor subtypes cases could have more than one matched control.
[c] Less than 20% of marker values were above lower limit of detection, results should be interpreted with caution.

patients was correlated with an impaired T cell response [19] and several small studies generally observed that circulating or peritoneal CRP levels were higher during post ovulatory phases of the menstrual cycle [20–24], indicating that CRP may be involved in the local wound healing process following ovulation. CRP remained the dominant risk factor as the associations for IL-8 and TNF α were attenuated after mutual adjustment for CRP.

Our study is the first to show an association between elevated circulating IL-1α and ovarian cancer risk; however, given that only 18.4% of values were above the LLOD for this marker, these results should be interpreted with caution. IL-1α is produced following nuclear factor kappa light chain enhancer of activated B cell (NF-κB) activation [25], and signaling of IL-1α through its receptor results in downstream activation of NF-κB [26], which leads to transcription of a number of genes whose products promote inflammation [27]. This pathway appears to play a crucial role in the process that links inflammation to cancer [28,29]. Specifically, activation of NF-κB through inhibitor of κB kinase epsilon (IKKε) was shown to be associated with more aggressive behavior in ovarian cancer cell lines [30] and has been associated with aberrant cellular activities in endometriosis, a known risk factor for ovarian cancer [31].

No previous study has shown an association between elevated circulating IL-8 and ovarian cancer using pre-diagnostic samples, the higher risk in serum samples collected in the most recent 5 years before diagnosis is consistent with evidence implicating the IL-8 pathway in later steps of carcinogenesis, including tumor progression and metastasis [32]. IL-8 has been shown to be elevated in ovarian cyst fluid, ascites, serum and tumor tissue from ovarian cancer patients and increased IL-8 expression correlates with poor prognosis and survival [33–39].

TNF α, like CRP, is a marker of various inflammation processes. TNF α has been shown to play a role in later steps of carcinogenesis [40,41]. For example, NF-κB activation by TNF α is involved in neoplastic transformation, proliferation, and tumor survival [42]. In addition, in ovarian cancer cells, TNF α enhances cell migration and metastasis through NF-κB dependent induction of IL-8, C-X-C chemokine receptor type 4 (CXCR4), monocyte chemoattractant protein 1 (MCP-1), and intercellular adhesion molecule 1 [43]. TNF α was positively associated with ovarian cancer in case-control studies using serum samples collected at diagnosis [19,44]. We report an increased risk of ovarian cancer with TNF α measured in pre-diagnostic serum. Our finding is not consistent with the null association reported by Clendenen et al. [45], however, the elevated, albeit not statistically significant, OR for

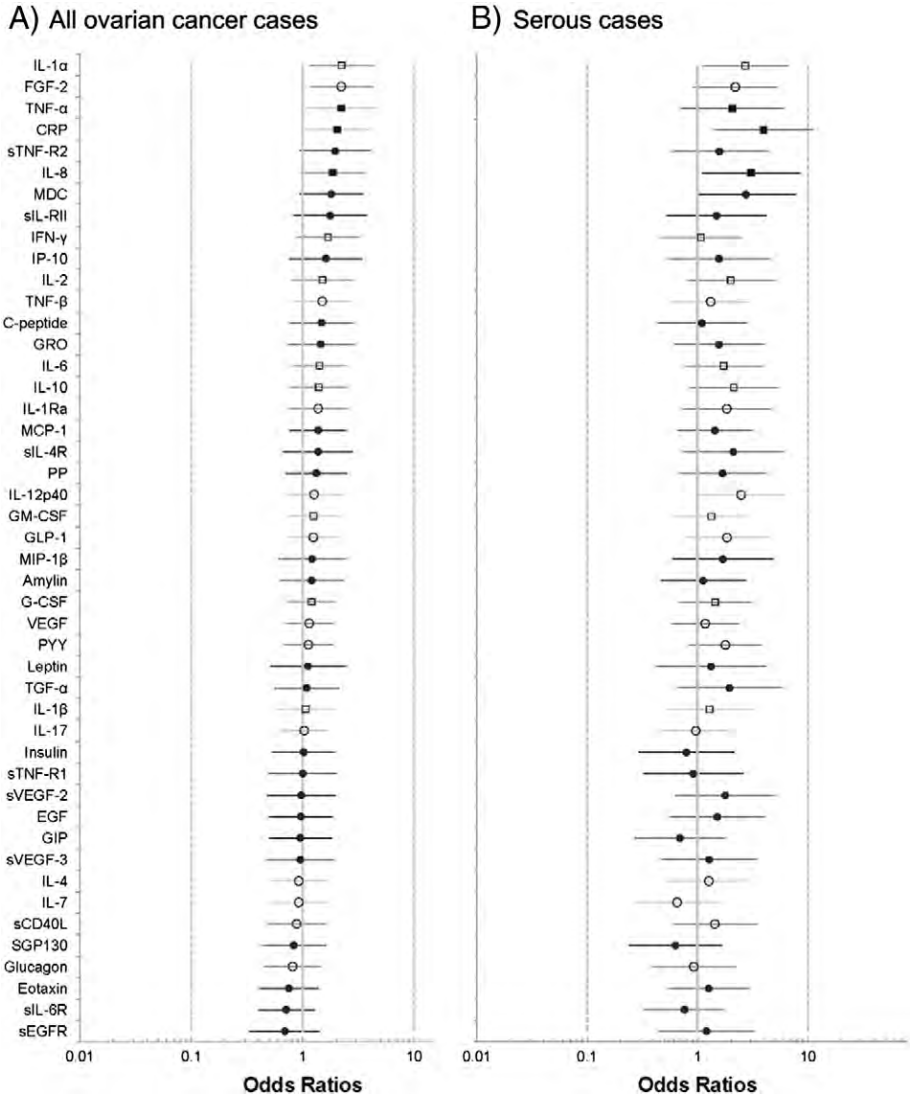

Fig. 1. Association between 46 inflammation markers and ovarian cancer risk using A) all ovarian cancer cases and B) serous ovarian cancer cases, nested case–control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. The symbol represents the odds ratio (OR) and the error bars represent the corresponding 95% confidence intervals. Filled markers indicate that the OR calculation was based on the comparison of individuals with marker measurements in Tertile 3 versus Tertile 1, unfilled markers indicate that the OR association is based on the comparison of individuals with marker measurements above the lower limit of detection (LLOD) versus values at or below LLOD. Square symbols indicate the 11 markers with a priori hypothesis regarding an association with ovarian cancer risk and circle symbols indicate the remaining 35 markers with weak or no prior evidence for an association.

TNF α receptor 2 observed in our study was consistent with the increased ORs reported by Poole et al. [7].

Inconsistent results in the existing studies may reflect limited case numbers in cohort studies that collected pre diagnostic specimens. Further, the use of different inflammation marker assays may have led to differing results across the studies. The multiplex assays utilized in the current study are comparable to those used by Clenenden et al. [45], however, the assay performance was noticeably different. For most of the inflammatory markers measured in the two studies, the percent of markers below LLOD was higher in the current study. Specifically, the low percent detection limited the ability to evaluate some markers [46] that were associated with ovarian cancer (i.e. IL 6 and IL 12p40) in the study by Clenenden et al. [45]. The assay performance in the current study was very similar to the systematic evaluation of multiplex inflammation marker panels published earlier by our group [17].

The strengths of our study include the prospective design, comprehensive evaluation of inflammation related markers measured using a validated technology, and careful control for confounding. We also note several limitations. Although we were able to include all ovarian cancer cases from the PLCO screening arm, the study was limited in power, which affected our ability to investigate associations with ovarian cancer subtypes other than serous tumors. Further, given the limited sample size, associations for all markers tested were imprecise. With respect to the inflammation hypothesis, however, the evidence is compelling for serous ovarian tumors, and many of the inflammation marker ovarian cancer associations strengthened in these analyses. While our observations support the association of pre diagnostic circulating markers of inflammation with ovarian cancer, they require replication given the large number of markers evaluated. Only the association with CRP and serous ovarian cancer was identified with an FDR less than 0.10. The associations between CRP, IL 1α, IL 8, TNF α and ovarian cancer had FDR q values of 0.13, while the remaining markers evaluated were not associated with ovarian cancer risk after correction for multiple comparisons. Further, we measured markers at only one time point; however, data suggests that most of the markers are moderately stable over time, with ICCs of 0.54 0.67 for CRP over four years [49,50], and an ICC of 0.87 for TNF α over three blood draws within two years [51]. In the only study published to date, the ICC for IL 8 was less stable (0.33 over two years) [51]. It is important to note that several markers of inflammation, namely CRP and TNF α, have also

Table 4
Mutually adjusted models of the association of CRP, IL-8 and TNF-α and ovarian cancer risk, nested case–control study in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial.

	All cases		Serous cases		Non-serous cases		Analyses of cases with specimens collected 2 to <5 years prior to diagnosis		Analyses of cases specimens collected 5+ years prior to diagnosis	
	n = 149		n = 83		n = 66		n = 93		n = 56	
	OR[a]	95% CI	OR[a]	95% CI	OR[a]	95% CI	OR[a]	95% CI	OR[a]	95% CI
CRP (mg/L)										
<3.23	1.00	Ref	1.00	Ref	1.00	Ref	1.00	Ref	1.00	Ref
3.23–9.76	1.38	0.71–2.65	1.63	0.56–4.75	2.90	0.86–9.84	1.41	0.63–3.17	1.53	0.41–5.74
>9.76	2.06	1.03–4.14	4.26	1.44–12.59	2.39	0.69–8.30	2.15	0.89–5.19	6.04	1.04–34.99
p-trend	0.046		0.007		0.32		0.10		0.041	
IL-8 (ng/L)										
<1.87	1.00	Ref	1.00	Ref	1.00	Ref	1.00	Ref	1.00	Ref
1.87–3.79	1.08	0.54–2.16	1.94	0.64–5.83	0.38	0.12–1.16	1.13	0.46–2.78	1.05	0.28–4.00
>3.80	1.78	0.88–3.60	3.66	1.22–11.03	0.93	0.24–3.52	2.26	0.87–5.85	1.52	0.40–5.83
p-trend	0.07		0.022		0.76		0.07		0.47	
TNF-α (ng/L)										
<4.05	1.00	Ref	1.00	Ref	1.00	Ref	1.00	Ref	1.00	Ref
4.05–5.48	1.72	0.89–3.34	0.88	0.34–2.25	6.99	1.82–26.88	1.09	0.48–2.49	6.24	1.43–27.32
>5.49	1.64	0.75–3.59	1.22	0.37–4.06	4.42	0.95–20.58	1.05	0.37–2.97	5.74	1.10–30.07
p-trend	0.24		0.75		0.10		0.92		0.049	

[a] Conditional logistic regression models adjusted for CRP, IL-8, TNF-α, body mass index, cigarette smoking status, duration of oral contraceptive use, and duration of menopausal hormone therapy use.

been associated with other tumors [46 48]. Presumably, these markers represent a common pathway of different inflammatory processes at different cancer sites. Future studies need to increase the focus on the tumor specific inflammatory mechanisms that underlie the reported associations of systemic inflammation markers here and in other studies. Lastly we note that the circulating inflammation markers measured in the current study may not reflect levels in local sites of inflammation relevant to ovarian carcinogenesis, which may include the fallopian tube, ovary or endometriotic lesions. Studies investigating the correlation between serum inflammation marker levels and different tissue types, using animal or human clinical specimens, could provide important insight into this question. As mentioned, additional research is needed to confirm these findings and better understand the role that inflammation may play in the etiology of ovarian cancer. If confirmed, further evaluation of these markers in risk prediction models is warranted.

In conclusion, our prospective investigation of 46 inflammation related markers provides evidence that serum levels of CRP and TNF α are associated with increased future risk of ovarian cancer, 5 or more years following blood collection. We also observed ovarian cancer associations for several novel markers that warrant further investigation. Increased inflammation may be etiologically important in ovarian carcinogenesis arguing for additional research to confirm and extend these findings.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2014.08.025.

Conflict of interest statement

The authors declare that they have no competing interests.

References

[1] Centers for Disease Control, Prevention. Ovarian cancer statistics; 2010 [Ref Type: Report].
[2] Ness RB, Cottreau C. Possible role of ovarian epithelial inflammation in ovarian cancer. J Natl Cancer Inst 1999;91:1459–67.
[3] Trabert B, Ness RB, Lo-Ciganic WH, Murphy MA, Goode EL, Poole EM, et al. Aspirin, nonaspirin nonsteroidal anti-inflammatory drug, and acetaminophen use and risk of invasive epithelial ovarian cancer: a pooled analysis in the Ovarian Cancer Association Consortium. J Natl Cancer Inst 2014;106:djt431.
[4] Kurman RJ, Shih I. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer — shifting the paradigm. Hum Pathol 2011;42:918–31.
[5] McSorley MA, Alberg AJ, Allen DS, Allen NE, Brinton LA, Dorgan JF, et al. C-reactive protein concentrations and subsequent ovarian cancer risk. Obstet Gynecol 2007;109:933–41.
[6] Lundin E, Dossus L, Clendenen T, Krogh V, Grankvist K, Wulff M, et al. C-reactive protein and ovarian cancer: a prospective study nested in three cohorts (Sweden, USA, Italy). Cancer Causes Control 2009;20:1151–9.
[7] Poole EM, Lee IM, Ridker PM, Buring JE, Hankinson SE, Tworoger SS. A prospective study of circulating C-reactive protein, interleukin-6, and tumor necrosis factor alpha receptor 2 levels and risk of ovarian cancer. Am J Epidemiol 2013;178:1256–64.
[8] Toriola AT, Grankvist K, Agborsangaya CB, Lukanova A, Lehtinen M, Surcel HM. Changes in pre-diagnostic serum C-reactive protein concentrations and ovarian cancer risk: a longitudinal study. Ann Oncol 2011;22:1916–21.
[9] Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. The biology of ovarian cancer. Semin Oncol 1998;25:281–304.
[10] Ziltener HJ, Maines-Bandiera S, Schrader JW, Auersperg N. Secretion of bioactive interleukin-1, interleukin-6, and colony-stimulating factors by human ovarian surface epithelium. Biol Reprod 1993;49:635–41.
[11] Norman RJ, Brannstrom M. Cytokines in the ovary: pathophysiology and potential for pharmacological intervention. Pharmacol Ther 1996;69:219–36.
[12] Espey LL. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. Biol Reprod 1994;50:233–8.
[13] Wilson JE. Adenocarcinomata in hens kept in a constant environment. Poult Sci 1958;37:1253.
[14] Godwin AK, Testa JR, Handel LM, Liu Z, Vanderveer LA, Tracey PA, et al. Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer. J Natl Cancer Inst 1992;84:592–601.
[15] Prorok PC, Andriole GL, Bresalier RS, Buys SS, Chia D, Crawford ED, et al. Design of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. Control Clin Trials 2000;21:273S–309S.
[16] Hayes RB, Sigurdson A, Moore L, Peters U, Huang WY, Pinsky P, et al. Methods for etiologic and early marker investigations in the PLCO trial. Mutat Res 2005;592:147–54.
[17] Chaturvedi AK, Kemp TJ, Pfeiffer RM, Biancotto A, Williams M, Munuo S, et al. Evaluation of multiplexed cytokine and inflammation marker measurements: a methodologic study. Cancer Epidemiol Biomarkers Prev 2011;20:1902–11.
[18] Vongpatanasin W, Tuncel M, Wang Z, Arbique D, Mehrad B, Jialal I. Differential effects of oral versus transdermal estrogen replacement therapy on C-reactive protein in postmenopausal women. J Am Coll Cardiol 2003;41:1358–63.
[19] Maccio A, Lai P, Santona MC, Pagliara L, Melis GB, Mantovani G. High serum levels of soluble IL-2 receptor, cytokines, and C reactive protein correlate with impairment of T cell response in patients with advanced epithelial ovarian cancer. Gynecol Oncol 1998;69:248–52.
[20] Blum CA, Muller B, Huber P, Kraenzlin M, Schindler C, De GC, et al. Low-grade inflammation and estimates of insulin resistance during the menstrual cycle in lean and overweight women. J Clin Endocrinol Metab 2005;90:3230–5.
[21] Capobianco G, de Muro P, Cherchi GM, Formato M, Lepedda AJ, Cigliano A, et al. Plasma levels of C-reactive protein, leptin and glycosaminoglycans during spontaneous menstrual cycle: differences between ovulatory and anovulatory cycles. Arch Gynecol Obstet 2010;282:207–13.
[22] Puder JJ, Blum CA, Mueller B, De GC, Dye L, Keller U. Menstrual cycle symptoms are associated with changes in low-grade inflammation. Eur J Clin Invest 2006;36:58–64.
[23] Wander K, Brindle E, O'Connor KA. C-reactive protein across the menstrual cycle. Am J Phys Anthropol 2008;136:138–46.

[24] Bouckaert PX, Evers JL, Doesburg WH, Schellekens LA, Brombacher PH, Rolland R. Patterns of changes in proteins in the peritoneal fluid of women during the periovulatory phase of the menstrual cycle. J Reprod Fertil 1986;77:329–36.
[25] Mori N, Prager D. Transactivation of the interleukin-1alpha promoter by human T-cell leukemia virus type I and type II Tax proteins. Blood 1996;87:3410–7.
[26] Bhat-Nakshatri P, Newton TR, Goulet Jr R, Nakshatri H. NF-kappaB activation and interleukin 6 production in fibroblasts by estrogen receptor-negative breast cancer cell-derived interleukin 1alpha. Proc Natl Acad Sci U S A 1998;95:6971–6.
[27] White KL, Rider DN, Kalli KR, Knutson KL, Jarvik GP, Goode EL. Genomics of the NF-kappaB signaling pathway: hypothesized role in ovarian cancer. Cancer Causes Control 2011;22:785–801.
[28] Greten FR, Arkan MC, Bollrath J, Hsu LC, Goode J, Miething C, et al. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. Cell 2007;130:918–31.
[29] Charbonneau B, Block MS, Bamlet WR, Vierkant RA, Kalli KR, Fogarty Z, et al. Risk of ovarian cancer and the NF-kappaB pathway: genetic association with IL1A and TNFSF10. Cancer Res 2014;74:852–61.
[30] Hsu S, Kim M, Hernandez L, Grajales V, Noonan A, Anver M, et al. IKK-epsilon coordinates invasion and metastasis of ovarian cancer. Cancer Res 2012;72:5494–504.
[31] Gonzalez-Ramos R, Defrere S, Devoto L. Nuclear factor-kappaB: a main regulator of inflammation and cell survival in endometriosis pathophysiology. Fertil Steril 2012;98:520–8.
[32] Yuan A, Chen JJ, Yao PL, Yang PC. The role of interleukin-8 in cancer cells and microenvironment interaction. Front Biosci 2005;10:853–65.
[33] Wang Y, Xu RC, Zhang XL, Niu XL, Qu Y, Li LZ, et al. Interleukin-8 secretion by ovarian cancer cells increases anchorage-independent growth, proliferation, angiogenic potential, adhesion and invasion. Cytokine 2012;59:145–55.
[34] Ivarsson K, Ekerydh A, Fyhr IM, Janson PO, Brannstrom M. Upregulation of interleukin-8 and polarized epithelial expression of interleukin-8 receptor A in ovarian carcinomas. Acta Obstet Gynecol Scand 2000;79:777–84.
[35] Penson RT, Kronish K, Duan Z, Feller AJ, Stark P, Cook SE, et al. Cytokines IL-1beta, IL-12, IL-6, IL-8, MCP-1, GM-CSF and TNFalpha in patients with epithelial ovarian cancer and their relationship to treatment with paclitaxel. Int J Gynecol Cancer 2000;10:33–41.
[36] Fasciani A, D'Ambrogio G, Bocci G, Luisi S, Artini PG, Genazzani AR. Vascular endothelial growth factor and interleukin-8 in ovarian cystic pathology. Fertil Steril 2001;75:1218–21.
[37] Kassim SK, El-Salahy EM, Fayed ST, Helal SA, Helal T, Azzam E, et al. Vascular endothelial growth factor and interleukin-8 are associated with poor prognosis in epithelial ovarian cancer patients. Clin Biochem 2004;37:363–9.
[38] Lokshin AE, Winans M, Landsittel D, Marrangoni AM, Velikokhatnaya L, Modugno F, et al. Circulating IL-8 and anti-IL-8 autoantibody in patients with ovarian cancer. Gynecol Oncol 2006;102:244–51.
[39] Nowak M, Glowacka E, Szpakowski M, Szyllo K, Malinowski A, Kulig A, et al. Proinflammatory and immunosuppressive serum, ascites and cyst fluid cytokines in patients with early and advanced ovarian cancer and benign ovarian tumors. Neuro Endocrinol Lett 2010;31:375–83.
[40] Balkwill F. Tumour necrosis factor and cancer. Nat Rev Cancer 2009;9:361–71.
[41] Wu Y, Zhou BP. TNF-alpha/NF-kappaB/Snail pathway in cancer cell migration and invasion. Br J Cancer 2010;102:639–44.
[42] Hsu TC, Nair R, Tulsian P, Camalier CE, Hegamyer GA, Young MR, et al. Transformation nonresponsive cells owe their resistance to lack of p65/nuclear factor-kappaB activation. Cancer Res 2001;61:4160–8.
[43] Kulbe H, Thompson R, Wilson JL, Robinson S, Hagemann T, Fatah R, et al. The inflammatory cytokine tumor necrosis factor-alpha generates an autocrine tumor-promoting network in epithelial ovarian cancer cells. Cancer Res 2007;67:585–92.
[44] Moradi MM, Carson LF, Weinberg B, Haney AF, Twiggs LB, Ramakrishnan S. Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in patients with ovarian epithelial cancer. Cancer 1993;72:2433–40.
[45] Clendenen TV, Lundin E, Zeleniuch-Jacquotte A, Koenig KL, Berrino F, Lukanova A, et al. Circulating inflammation markers and risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev 2011;20:799–810.
[46] Shiels MS, Pfeiffer RM, Hildesheim A, Engels EA, Kemp TJ, Park JH, et al. Circulating inflammation markers and prospective risk for lung cancer. J Natl Cancer Inst 2013;105:1871–80.
[47] Il'yasova D, Colbert LH, Harris TB, Newman AB, Bauer DC, Satterfield S, et al. Circulating levels of inflammatory markers and cancer risk in the health aging and body composition cohort. Cancer Epidemiol Biomarkers Prev 2005;14:2413–8.
[48] Purdue MP, Lan Q, Bagni R, Hocking WG, Baris D, Reding DJ, et al. Prediagnostic serum levels of cytokines and other immune markers and risk of non-hodgkin lymphoma. Cancer Res 2011;71:4898–907.
[49] Glynn RJ, MacFadyen JG, Ridker PM. Tracking of high-sensitivity C-reactive protein after an initially elevated concentration: the JUPITER Study. Clin Chem 2009;55:305–12.
[50] Pischon T, Hankinson SE, Hotamisligil GS, Rifai N, Rimm EB. Leisure-time physical activity and reduced plasma levels of obesity-related inflammatory markers. Obes Res 2003;11:1055–64.
[51] Epstein MM, Breen EC, Magpantay L, Detels R, Lepone L, Penugonda S, et al. Temporal stability of serum concentrations of cytokines and soluble receptors measured across two years in low-risk HIV-seronegative men. Cancer Epidemiol Biomarkers Prev 2013;22:2009–15.